

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION

Unclassified

1b. RESTRICTIVE MARKINGS

3. DISTRIBUTION/AVAILABILITY OF REPORT

Distribution unlimited - approved for public release

5. MONITORING ORGANIZATION REPORT NUMBER(S)

AD-A208 795

FILE

ER(S)

6a. NAME OF PERFORMING ORGANIZATION

U.S. Army Medical Research
Institute of Infectious Diseases

6b. OFFICE SYMBOL

(if applicable)

SGRD-UIP-B

7a. NAME OF MONITORING ORGANIZATION

U.S. Army Medical Research and Development
Command

6c. ADDRESS (City, State, and ZIP Code)

Ft. Detrick, Frederick, MD 21701-5011

7b. ADDRESS (City, State, and ZIP Code)

Ft. Detrick, Frederick, MD 21701-5012

8a. NAME OF FUNDING/SPONSORING
ORGANIZATION8b. OFFICE SYMBOL
(if applicable)

9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER

8c. ADDRESS (City, State, and ZIP Code)

10. SOURCE OF FUNDING NUMBERS

PROGRAM
ELEMENT NO.PROJECT
NO.TASK
NO.WORK UNIT
ACCESSION NO.

11. TITLE (Include Security Classification)

Effects of Divalent Cations on Snake Venom Cardiotoxin-Induced Hemolysis and ³H-Deoxyglucose-6-Phosphate Release from Human Red Blood Cells

12. PERSONAL AUTHOR(S)

Drs. Ming-Shi Jiang, Jeffrey E. Fletcher and Leonard A. Smith

13a. TYPE OF REPORT

13b. TIME COVERED

FROM _____ TO _____

14. DATE OF REPORT (Year, Month, Day)

24 MAY 89

15. PAGE COUNT

22

16. SUPPLEMENTARY NOTATION

17. COSATI CODES

FIELD

GROUP

SUB-GROUP

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Effects of divalent cations on snake venom cardiotoxin-induced hemolysis and ³H-deoxyglucose-6-phosphate release from human red blood cells. At a low concentration of Naja naja kaouthia cardiotoxin (3 μM), Ca²⁺, Sr²⁺ and Ba²⁺ (2 mM), had little to no effect on ³H-deoxyglucose-6-phosphate (³H-dGlu-6-p) or hemoglobin release. At higher concentrations of N. n. kaouthia cardiotoxin (>10 μM), Ca²⁺ (2 mM), but not Sr²⁺ or Ba²⁺, significantly enhanced ³H-dGlu-6-p and hemoglobin release. Mn²⁺ (2 mM) almost completely inhibited ³H-dGlu-6-p release and hemolysis at both the 3 μM and 10 μM concentrations of cardiotoxin. At a fixed concentration of N. n. kaouthia cardiotoxin (3 μM), Ca²⁺ at low concentrations (0.5 mM) enhanced ³H-dGlu-6-p and hemoglobin release, but at higher concentrations caused a dose-dependent inhibition of cardiotoxin action. The cardiotoxin from N. n. kaouthia venom (3 μM) induced ³H-dGlu-6-p release and hemolysis release with similar time courses and to similar extents. ³H-dGlu-6-p release induced by cardiotoxin was greatly enhanced as the pH of the medium was increased from 7.0 to 8.5. Similarities between ³H-dGlu-6-p and hemoglobin release do not support opening of pores in the plasmalemma of all red blood cells as the mode of action of cardio-

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT

☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS

21. ABSTRACT SECURITY CLASSIFICATION

22a. NAME OF RESPONSIBLE INDIVIDUAL

22b. TELEPHONE (Include Area Code)

22c. OFFICE SYMBOL

Effects of Divalent Cations on Snake Venom Cardiotoxin-Induced Hemolysis
and ^3H -Deoxyglucose-6-Phosphate Release from Human Red Blood Cells

Ming-Shi Jiang,¹ Jeffrey E. Fletcher^{1*} and Leonard A. Smith²

¹Department of Anesthesiology, Hahnemann University, Philadelphia, PA, 19102-1192 U.S.A.

²Department of Toxinology, Pathology Division, USAMRIID, Fort Detrick, MD 21701, U.S.A.

Running Title: Divalent cations and CTX action

Send Correspondence and Reprint Requests to:

Jeffrey E. Fletcher, Ph.D.
Department of Anesthesiology MS-310
Hahnemann University
Broad and Vine Streets
Philadelphia, PA 19102-1192
U.S.A.



Accession For	
NTIS GRA&I	<input checked="" type="checkbox"/>
DTIC TAB	<input type="checkbox"/>
Unannounced	<input type="checkbox"/>
Justification	
By	
Distribution/	
Availability Codes	
Dist	Avail and/or Special
A-1	

*Author to whom correspondence should be addressed.

80 3 C7 004

ABSTRACT

Jiang, M-S, Fletcher, J.E. and Smith, L.A. Effects of divalent cations on snake venom cardiotoxin-induced hemolysis and ^3H -deoxyglucose-6-phosphate release from human red blood cells. Toxicon __, __-__, 19__.

At a low concentration of Naja naja kaouthia cardiotoxin ($3\ \mu\text{M}$) Ca^{2+} , Sr^{2+} and Ba^{2+} (2 mM), had little to no effect on ^3H -deoxyglucose-6-phosphate (^3H -dGlu-6-p) or hemoglobin release. At higher concentrations of N. n. kaouthia cardiotoxin ($\geq 10\ \mu\text{M}$), Ca^{2+} (2 mM), but not Sr^{2+} or Ba^{2+} , significantly enhanced ^3H -dGlu-6-p and hemoglobin release. Mn^{2+} (2 mM) almost completely inhibited ^3H -dGlu-6-p release and hemolysis at both the $3\ \mu\text{M}$ and $10\ \mu\text{M}$ concentrations of cardiotoxin. At a fixed concentration of N. n. kaouthia cardiotoxin ($3\ \mu\text{M}$), Ca^{2+} at low concentrations (0.5 mM) enhanced ^3H -dGlu-6-p and hemoglobin release, but at higher concentrations caused a dose-dependent inhibition of cardiotoxin action. The cardiotoxin from N. n. kaouthia venom ($3\ \mu\text{M}$) induced ^3H -dGlu-6-p release and hemolysis release with similar time courses and to similar extents. ^3H -dGlu-6-p release induced by cardiotoxin was greatly enhanced as the pH of the medium was increased from 7.0 to 8.5. Similarities between ^3H -dGlu-6-p and hemoglobin release do not support opening of pores in the plasmalemma of all red blood cells as the mode of action of cardiotoxins, but suggest that complete lysis of a subpopulation of cells occurs. Cardiotoxins have two components of lysis, only one of which is Ca^{2+} -dependent. The Ca^{2+} -dependent lysis is only evident at higher cardiotoxin concentrations. Mn^{2+} is an effective antagonist of cardiotoxin action.

INTRODUCTION

Snake venom cardiotoxins (CTXs) are generally regarded as low molecular weight basic polypeptides that arrest the heart in systole (Sarkar, 1951; Lee *et al.*, 1968), depolarize and induce contractures in skeletal muscle (Chang, 1979) and hemolyze red blood cells (Condrea, 1974; 1979). The specific mechanism of action of the CTXs is not clear. Divalent cations have varied effects on CTX-induced hemolysis. In general, Ca^{2+} and Sr^{2+} at concentrations $> 2 \text{ mM}$ in the bathing medium have an inhibitory effect on hemolysis by low concentrations ($\leq 3 \text{ }\mu\text{M}$) of snake venom CTXs and lower concentrations of Ca^{2+} can have slight stimulatory effects (Jiang *et al.*, 1989). At higher concentrations of CTX ($\geq 10 \text{ }\mu\text{M}$), hemolysis appears to be enhanced by physiologically relevant levels of Ca^{2+} (2 mM) in the bathing medium, but not by Sr^{2+} or Ba^{2+} (Jiang *et al.*, 1989). These effects of divalent cations have not been examined as regards ^3H -deoxyglucose-6-phosphate (^3H -dGlu-6-p) release. Mn^{2+} can reverse the cardiotoxic action of a phospholipase A_2 (PLA $_2$) with CTX-like properties from *Naja nigricollis* snake venom (Fletcher *et al.*, 1982). The effects of Mn^{2+} on the hemolytic action of typical cobra CTXs have not been examined.

Hemolysis may be the expression of red blood cell membrane destruction by snake venom factors, including CTXs (Condrea, 1979). Considering that hemolysis of fresh human red blood cells by CTXs is usually lower than 30%, either complete lysis takes place only in a subpopulation of cells in an all-or-none manner, or the entire population of cells undergoes only partial lysis through membrane pores. Therefore, we compared hemoglobin release to release of a much smaller marker (^3H -dGlu-6-p) of toxin action to determine if considerably greater leakage of ^3H -dGlu-6-p would occur, thus supporting a

"pore" theory. Radiolabeled 2-deoxy-D-glucose (dGlu) is taken up into cells by the same transport system as D-glucose and is phosphorylated to 2-deoxy-D-glucose-6-p (d-Glu-6-p) by hexokinase (Smith and Gorski, 1968). Since d-Glu-6-p is a metabolically inert compound (Smith and Gorski, 1968; Kipnis and Cori, 1959), and cell membrane permeability is low for d-Glu-6-p, this molecule accumulates inside the cell (Walum and Peterson, 1982). The high level of accumulated intracellular radioactivity and the comparatively small size of ^3H -dGlu-6-p make it a sensitive probe for cell membrane permeability studies (Walum and Peterson, 1982).

In the present study we examined ^3H -dGlu-6-p release and hemolysis under a variety of conditions to test the "pore" hypothesis. Divalent cations were used in these studies, as they have varied effects on CTX-induced hemolysis, but have not been examined in relation to ^3H -dGlu-6-p release. Mn^{2+} , in particular, was examined as an antagonist of CTX action.

MATERIALS AND METHODS

Materials. CTX from N. n. kaouthia venom (lot no. 125F-4007) was purchased from Sigma Chemical Company (St. Louis, MO) and used without further purification. CTX from Naja naja atra venom was purified from venom by fast high performance liquid chromatography with ionic exchange chromatography, as described in the Results section. These two cobra CTXs were highly purified and relatively free from PLA₂ contamination, as previously reported (Jiang et al., 1989). The PLA₂ with CTX-like properties from Bungarus fasciatus venom was purified as previously described (Ji et al., 1983). Human red blood cells were obtained from the American Red Cross Blood Service as packed cells derived from CPD whole blood (AS-1) and were stored at 4°C. The red blood cells were used within two weeks of their expiration date. Radiolabeled 2-deoxy-D-(1-³H)-glucose (15-25 Ci/mMole) was purchased from Amersham (U.K.).

Methods. ³H-d-Glu-6-p release from erythrocytes was determined as previously described (Jiang et al., 1987). Packed red blood cells were washed three times with N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES)-buffered preloading solution (HEPES 20 mM, NaCl 130 mM pH 7.4) The packed erythrocytes pellets (2 ml) were added to 2 ml of preloading solution and then were incubated with ³H-dGlu (5 µCi) for 3 hr at 37°C. The loaded cells were then washed three times with cold (4°C) preloading solution. Aliquots (4 µl) of preloaded cells were added to 200 µl of incubation solution (preloading solution containing 5 mM glucose and indicated divalent cation) and incubated at 37°C for the indicated time. The tubes were centrifuged and 200 µl supernatant was added to 3 ml of the complete counting cocktail (RPI; Prospect, IL). Radioactivity was determined with a Beckman scintillation counter (Model LS 5801). The 100% ³H-dGlu-6-p release was determined by

incubating 4 μ l of erythrocytes in 200 μ l of H₂O. Hemolysis of human erythrocytes was performed as previously described (Jiang *et al.*, 1989). Before incubation with CTX, erythrocytes were washed three times (HEPES 20mM NaCl 130 mM pH 7.4). Following the final centrifugation, a 4 μ l aliquot of packed red blood cells was added to 200 μ l incubation medium (like the ³H-dGlu-6-p release experiment) then incubated at 37°C for the indicated time, centrifuged and the hemoglobin release estimated by reading the absorbance of the supernatant at 540 nm. The 100% value for hemoglobin release was determined by adding 4 μ l of erythrocytes to 200 μ l distilled water.

RESULTS

Venom from *N. n. atra* was resuspended in 5 mM sodium acetate, pH 5.8, filtered and 200 mg aliquots were fractionated on a Mono S preppacked HR 10/10 column from Pharmacia, Inc. (Piscataway, NJ). The column was developed with an increasing sodium acetate gradient from 0.005 M (pH 5.8) to 0.5 M (pH 6.5) (Fig. 1A). Fractions 75-78, representing CTX II, were pooled, dialyzed against 50 mM 2-[N-morpholino]ethanesulfonic acid (MES), pH 6.5, and recycled on a second Mono S column. The column was developed with an increasing NaCl gradient from 0 to 1 M NaCl in 50 mM MES (pH 6.5) (Fig. 1B). Fractions 12-14 from this second column were pooled, dialyzed against 50 mM MES pH 6.5, and lyophilized. The *N. n. atra* CTX fraction appeared as a single band of about 7,000 MW on sodium dodecyl sulfate polyacrylamide gel electrophoresis slab gels.

The two CTXs and the PLA₂ with CTX-like properties differ in hemolytic potency [*N. n. kaouthia* CTX > *N. n. atra* CTX >> *B. fasciatus* PLA₂ (Jiang *et al.*, 1989)]. We examined whether the three toxins exhibit a similar order of potency in eliciting the release of a considerably smaller molecule (³H-dGlu-6-p). Indeed, the order of potency for ³H-dGlu-6-p release from human red blood cells was *N. n. kaouthia* (Fig. 2A) > *N. n. atra* (Fig. 2B) >> *B. fasciatus* toxin (Fig. 2C). The same order of potency was observed in Ca²⁺-containing or Ca²⁺-free media (Fig. 2). Extracellular Ca²⁺ (2 mM) enhanced the hemolytic (Jiang *et al.*, 1989) and ³H-dGlu-6-p releasing (Fig. 2) activities of *N. n. kaouthia* and *N. n. atra* CTXs, but only at concentrations of CTX ≥ 10 μM. These effects of Ca²⁺ were less evident for the weakly hemolytic *B. fasciatus* PLA₂ with CTX-like properties. We compared the effects of five different divalent cation conditions (none, Ca²⁺, Sr²⁺, Ba²⁺, Mn²⁺) on

hemolysis and ^3H -dGlu-6-p release at two concentrations of *N. n. kaouthia* CTX (Fig. 3). At a lower concentration (3 μM) of *N. n. kaouthia* CTX both ^3H -dGlu-6-p release and hemolysis in Ca^{2+} , Sr^{2+} or Ba^{2+} medium were similar, or only slightly lower, than in divalent cation free medium. However, at a higher concentration (10 μM) of *N. n. kaouthia* CTX both ^3H -dGlu-6-p release and hemolysis were greatly enhanced in a Ca^{2+} containing medium, but not in Sr^{2+} or Ba^{2+} containing media (Fig. 3). Both ^3H -dGlu-6-p release and hemolysis were almost completely inhibited in Mn^{2+} (2 mM) containing medium at either 3 or 10 μM *N. n. kaouthia* CTX (Fig. 3). To avoid the general stimulatory effect of Ca^{2+} at higher concentrations of CTX (Fig. 2), the effects of varying the concentration of Ca^{2+} on ^3H -dGlu-6-p release and hemolysis were examined at a fixed *N. n. kaouthia* CTX concentration of 3 μM . Low concentrations of Ca^{2+} (<1 mM) enhanced ^3H -dGlu-6-p release and, to a greater extent, hemolysis relative to that in cation free media (Fig. 4). Beyond a Ca^{2+} concentration of 2 mM, both ^3H -dGlu-6-p release and hemolysis were inhibited by Ca^{2+} in a dose-dependent manner (Fig. 4). As with hemolysis (Jiang *et al.*, 1989), ^3H -dGlu-6-p release induced by *N. n. kaouthia* CTX (3 μM) exhibits a sharp increase in the range of pH 7.0 to 8.5 (Fig. 5). We compared the time course of leakage of this large molecule to that of a considerably smaller molecule (^3H -dGlu-6-p) to determine if ^3H -dGlu-6-p release is a more sensitive indicator of CTX action. While ^3H -dGlu-6-p release appeared to be detectable at a slightly earlier time than hemolysis, there were no dramatic differences in the time courses of release of these two markers of CTX action (Fig. 6).

DISCUSSION

Divalent cations have varied effects on snake venom CTX action that are dependent on the concentration of toxin and the type and concentration of divalent cation. At low concentrations of toxin ($\leq 3 \mu\text{M}$), divalent cations have only a slight stimulatory effect at concentrations $< 2 \text{ mM}$ and dramatic dose-response related inhibitory effects $> 2 \text{ mM}$. However, at higher ($\geq 10 \mu\text{M}$) concentrations of toxin, a very different effect of divalent cations was observed. At these higher concentrations of toxin, only Ca^{2+} (2 mM) stimulated hemolysis or ^3H -dGlu-6-p release, not Sr^{2+} or Ba^{2+} . This effect of Ca^{2+} may depend to some extent on the specific CTX, as ^3H -dGlu-6-p (Fig. 2) and hemoglobin (Jiang *et al.*, 1989) release are both stimulated by Ca^{2+} to a greater extent when induced by the *N. n. kaouthia* CTX than by the *N. n. atra* CTX. Mn^{2+} was a potent inhibitor of CTX action at low and high concentrations of toxin. This observation is in agreement with a previous study in a rat atrial preparation in which the depolarizing and action potential blocking effects of a PLA₂ with CTX-like activity were antagonized and reversed by Mn^{2+} (Fletcher *et al.*, 1982). Thus, understanding the mechanism of Mn^{2+} action might be important in elucidating the action of the cobra venom CTXs.

CTX and PLA₂ act synergistically to induced hemolysis of red blood cells (Condrea, 1974; 1979). It is difficult to purify CTX fractions that are free of PLA₂ contamination (Louw and Visser, 1978). Snake venom PLA₂ is a Ca^{2+} -dependent enzyme (Iwanaga and Suzuki, 1979). Therefore, it is possible that the enhanced hemolytic activity of high concentrations of the CTXs in Ca^{2+} medium is due to activation by Ca^{2+} of trace amounts of PLA₂ contaminating the toxin fraction. It is also possible that the antagonistic effect of Mn^{2+} on hemolytic activity is due to inhibition of this trace PLA₂ activity. We have

previously reported that our *N. n. kaouthia* and *N. n. atra* CTX fractions are relatively free of PLA₂ contamination (<0.0001%) as determined by titration methods (Dole, 1956) using mixed micelle substrates of Triton X-100 and egg yolk phosphatidylcholine (Jiang *et al.*, 1989). Using more sensitive approaches, such as gas chromatographic analysis of free fatty acids produced by the CTX fractions in biological membranes (erythrocytes), or PLA₂ activity of the CTX fractions on radiolabeled substrates (phosphatidylcholine; ¹⁴C- β -arachidonyl), we have observed very low levels of PLA₂ activity in these fractions (unpublished observations). The trace PLA₂ activity on radiolabeled substrates is reduced by about 95% following treatment of the CTX fraction with p-bromophenacyl bromide (unpublished observations), suggesting that it is due to contamination with venom PLA₂ and is not intrinsic to the CTX, which lacks the histidine residue (*Naja naja siamensis*; Dimari *et al.*, 1975). Since the same p-bromophenacyl bromide treatment has no effect on the hemolytic activity of the *N. n. kaouthia* CTX (100 μ M) in a Ca²⁺ containing medium (Jiang *et al.*, 1989), it is unlikely that this low level of contamination of the CTX fractions with PLA₂ has any relationship to hemolytic activity or ³H-dGlu-6-p release in the present study.

The relatively low levels of either ³H-dGlu-6-p or hemoglobin release suggest that either: 1) only a subpopulation of cells is affected by the toxin and this is an all-or-none phenomenon, or; 2) all of the cells exposed to the toxin undergo only partial lysis. The similarities between ³H-dGlu-6-p and hemoglobin release, however, suggest that the second possibility is unlikely. If all the cells underwent partial lysis (slight opening of the leakage "pores"), then ³H-dGlu-6-p release would exceed hemoglobin release, as the smaller marker would be released at much greater amount than the larger

marker. What population of cells would most likely be susceptible to lysis? Other investigators have reported that young red blood cells are resistant to hemolysis relative to aged cells when both populations are isolated from fresh human red blood cells (Chen et al., 1984). In vitro aging of red blood cells also leads to considerably greater levels of hemolysis by CTXs (Jiang et al., 1989). Therefore, the older blood cell population is most likely the subpopulation susceptible to lysis by CTXs.

Acknowledgements. This research supported in part by the U.S. Army Medical Research and Development Command contract no. DAMD17-87-C-7155.

REFERENCES

- Chang, C.C. (1979) The action of snake venoms on nerve and muscle. In Snake Venoms pp. 309-358 (Lee, C.Y. Ed) Berlin, Springer-Verlag.
- Chen, Y-H, Hu, C-T, Yang, J.T. (1984) Membrane disintegration and hemolysis of human erythrocytes by snake venom cardiotoxin (a membrane-disruptive polypeptide). Biochem. Int. 8, 329-338.
- Condrea, E. (1974) Membrane-active polypeptides from snake venom: cardiotoxins and haemocytotoxins. Experientia 30, 121-129.
- Condrea, E. (1979) Hemolytic effects of snake venom. In Snake Venoms pp. 448-471 (Lee, C.Y. Ed) Berlin, Springer-Verlag.
- Dimari, S.J., Lembach, K.J. and Chatman, V.B. (1975) The cytotoxins of cobra venoms: Isolation and partial characterization. Biochim. biophys. Acta 393, 320-334.
- Dole, V.P. (1956) A relation between non-esterified fatty acids in plasma and the metabolism of glucose. J. clin. Invest. 35, 150-154.
- Fletcher, J.E., Yang, C.C. and Rosenberg, P. (1982) Basic phospholipase A₂ from Naja nigricollis snake venom: Phospholipid hydrolysis and effects on electrical and contractile activity of the rat heart. Toxicol. Appl. Pharmacol. 66, 39-54.
- Iwanaga, S. and Suzuki, T. (1979) Enzymes in snake venoms. In Snake Venoms pp. 61-158 (Lee, C.Y. Ed) Berlin, Springer-Verlag.
- Ji, Y., Xue, T., Xu, Y., Jiang, M-S. and Xu, K. (1983) Isolation and identification of cardiotoxic and neurotoxic components from venom of Bungarus fasciatus schneider. Acta Herpetologica Sinica 2, 69-74.
- Jiang, M., Häggblad, J., Heilbronn, E., Rydqvist, B. and Eaker, D. (1987) Some biochemical characteristics and cell membrane actions of a toxic

phospholipase A₂ isolated from the venom of the pit viper *Agkistrodon halys* (Pallas). *Toxicon* 25, 785-792.

Jiang, M.S., Fletcher, J.E. and Smith, L.A. (1989) Factors influencing the hemolysis of human erythrocytes by cardiotoxins from *Naja naja kaouthia* and *Naja naja atra* venoms and a phospholipase A₂ with cardiotoxin-like properties from *Bungarus fasciatus* venom. *Toxicon* 27, 247-257.

Kipnis, D.M., and Cori, C.F. (1959) Studies of tissue permeability V. The penetration and phosphorylation of 2-deoxyglucose in the rat diaphragm. *J. Biol. Chem.* 243, 171-177.

Lee, C.Y., Chang, C.C., Chiu, T.H., Tseng, T.C. and Lee, S.Y. (1968) Pharmacological properties of cardiotoxin isolated from Formosan cobra venom. *Naunyn-Schmiedebergs Arch. Pharmacol.* 259, 360-374.

Louw, A.I. and Visser, L. (1978) The synergism of cardiotoxin and phospholipase A₂ in hemolysis. *Biochim. biophys. Acta* 512, 163-171.

Sarkar, N.K. (1951) Action mechanism of cobra venom, cardiotoxin and allied substances on muscle contraction. *Proc. Soc. Exp. Biol.*, 78, 469-471.

Smith, D.E. and Gorski, J. (1968) Estrogen control of uterine glucose metabolism 1. An analysis based on the transport and phosphorylation of 2-deoxyglucose. *J. Biol. Chem.* 243, 4169-4174.

Walum, E. and Peterson, A. (1982) Tritiated 2-Deoxy-D-glucose as a probe for cell membrane permeability studies. *Analyt. Biochem.* 120, 8-11.

FIGURE LEGENDS

- Fig. 1. Purification of CTX II from *N. n. atra* venom. (A) Chromatography of *N. n. atra* venom. Aliquots (200 mg) of venom were fractionated on an FPLC Mono S prepacked HR 10/10 column. The column was developed with an increasing sodium acetate gradient from 0.005 M (pH 5.8) to 0.5 M (pH 6.5). Fractions 70 to 95 contained CTXs I, II, III and IV. (B) Chromatography of *N. n. atra* CTX II. Fractions 75 to 78 from the first Mono S column were pooled, dialyzed against MES (50 mM), pH 6.5 and recycled on a second FPLC Mono S prepacked with HR 10/10 column. The column was developed with an increasing NaCl gradient from 0 to 1 M in MES (50 mM), pH 6.5. Fractions 12-14 were pooled and dialyzed against MES (50 mM), pH 6.5. These fractions, representing CTX II, yielded a single band of about 7000 MW when analyzed on 8 to 25% SDS-PAGE.
- Fig. 2. Dose response curve of ^3H -dGlu-6-p release induced by CTXs from *N. n. kaouthia* (A) and *N. n. atra* (B) venoms and a PLA₂ with CTX-like properties from *B. fasciatus* venom (C) in Ca^{2+} free (unfilled circles) or 2 mM Ca^{2+} (filled circles) media. Red blood cells were preloaded with ^3H -dGlu, and incubated with the toxins for 2 hr at pH 7.4 and 37°C. Values are the mean of triplicate determinations. The maximum SD was 2.6% at 30 μM and 1.2% at lower toxin concentrations.

Fig. 3. The effect of divalent cations and CTX concentration on hemolysis and ^3H -dGlu-6-p release induced by *N. n. kaouthia* CTX. Erythrocytes were incubated with the CTX for 2 hr at pH 7.4 and 37°C in a buffer containing a 2 mM concentration of Ca^{2+} , Sr^{2+} , Ba^{2+} , Mn^{2+} , or not containing divalent cation. (A) *N. n. kaouthia* CTX (3 μM). (B) *N. n. kaouthia* CTX (10 μM). Values are the mean of triplicate determinations and the bars indicate SD. Analysis of variance and Newman-Keuls test were used to test those conditions different from a divalent cation-free medium (* $p < 0.05$; ** $p < 0.01$).

Fig. 4. Effects of Ca^{2+} concentration in a range of 0-40 mM on hemolysis and ^3H -dGlu-6-p release by *N. n. kaouthia* CTX (3 μM). Erythrocytes were incubated with the CTX for 2 hr at pH 7.4 and 37°C and hemolysis (unfilled circles) or ^3H -dGlu-6-p release (filled circles) determined. Values are the mean of triplicate determinations. The maximum SD was 1.2%.

Fig. 5. The effects of pH on ^3H -dGlu-6-p release by *N. n. kaouthia* CTX (3 μM). Erythrocytes were incubated with the CTX for 2 hr at the indicated pH and 37°C. Values are the mean of triplicate determinations. The maximum SD was <1.0%. A divalent cation-free incubation medium was used.

Fig. 6. Time course of ^3H -dGlu-6-p release and hemolysis by *N. p. kaouthia* CTX (3 μM). Erythrocytes were incubated with the CTX for the indicated time at pH 7.4 and 37°C and hemolysis (unfilled circles) or ^3H -dGlu-6-p release (filled circles) determined. Values are mean of triplicate determinations. The maximum SD was <1.0%. A divalent cation-free incubation medium was used.

Figure 1

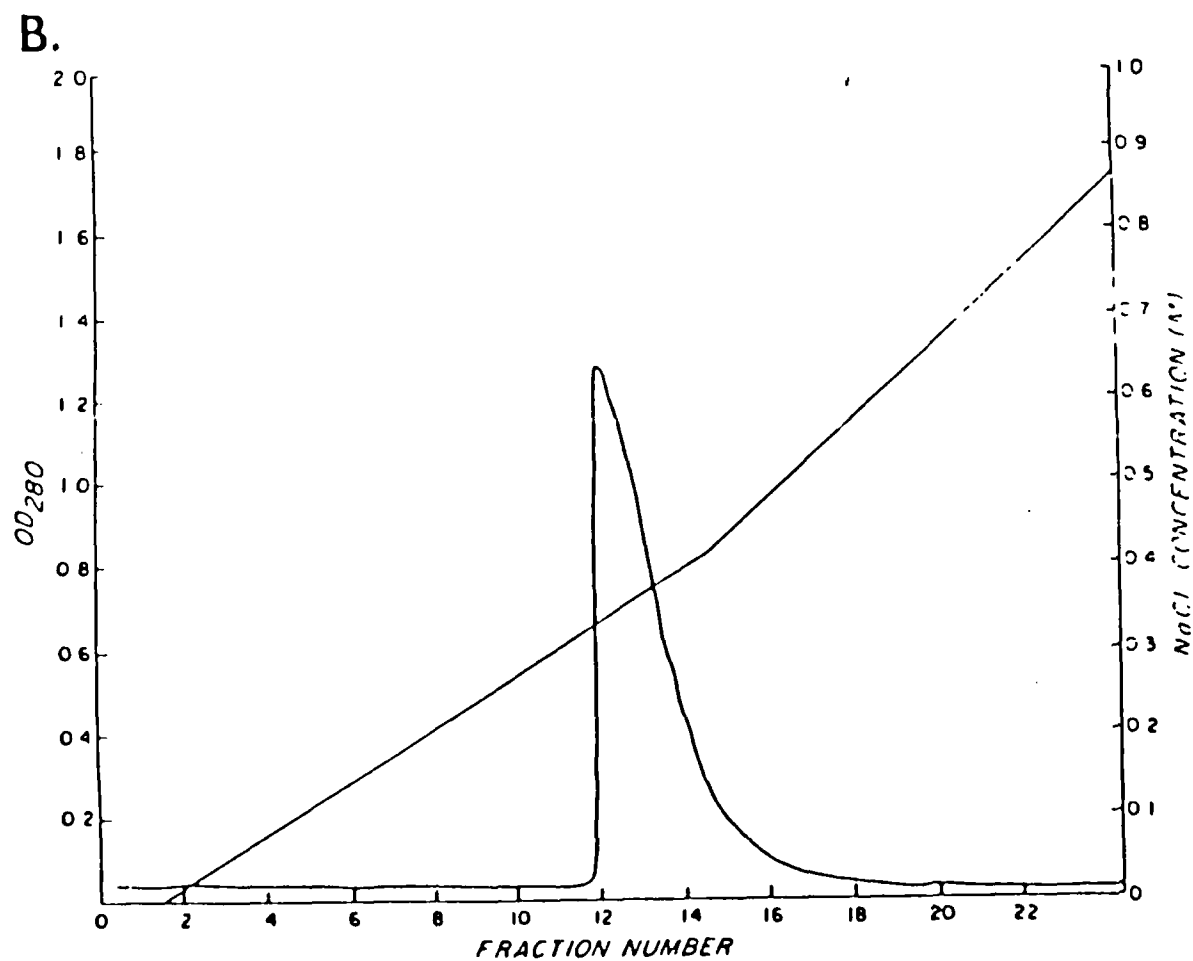
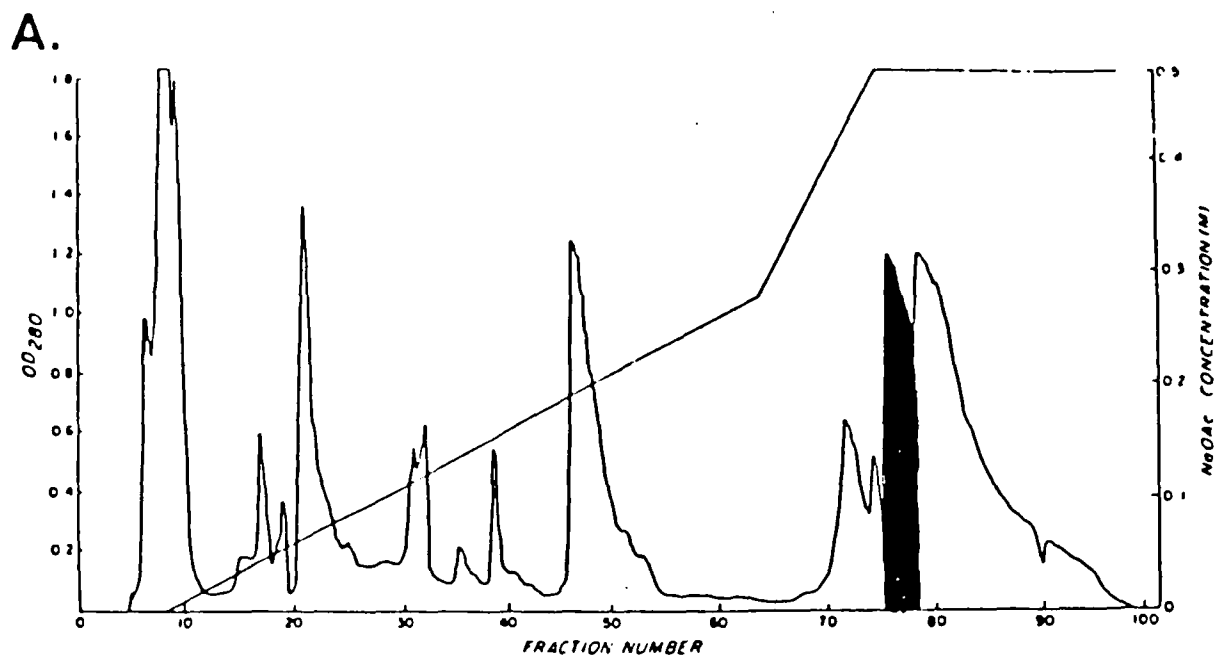


Figure 2

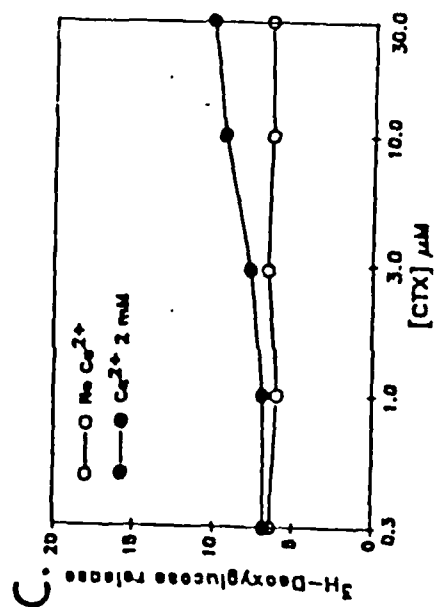
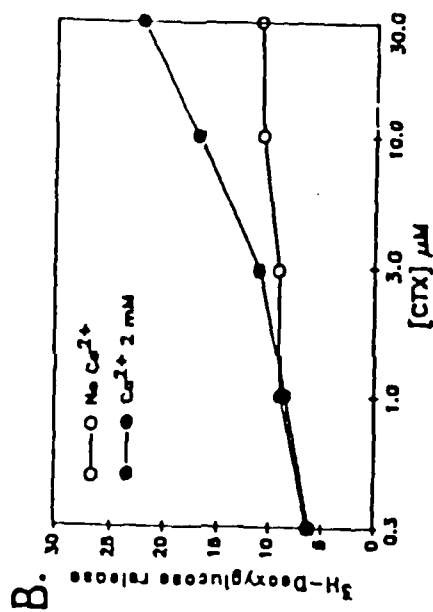
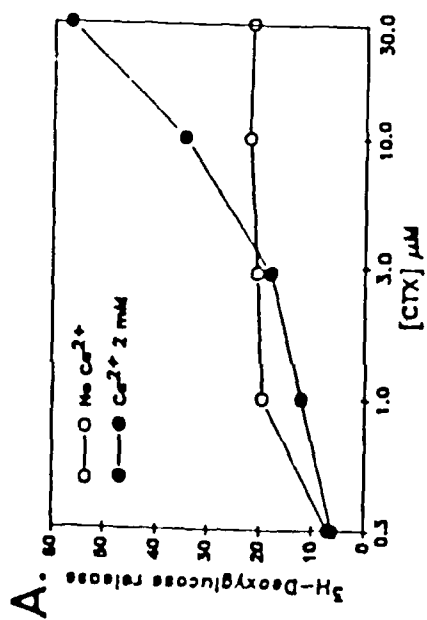


Figure 3

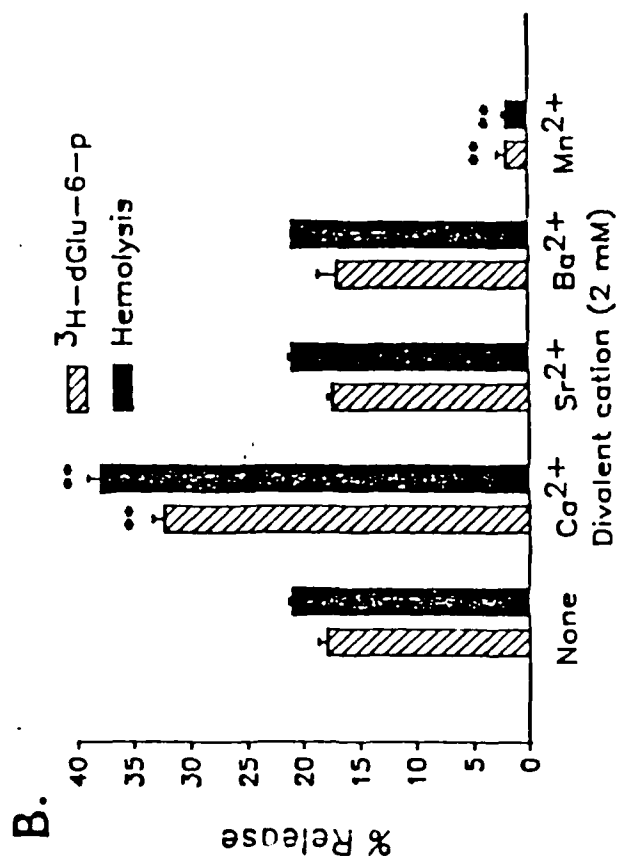
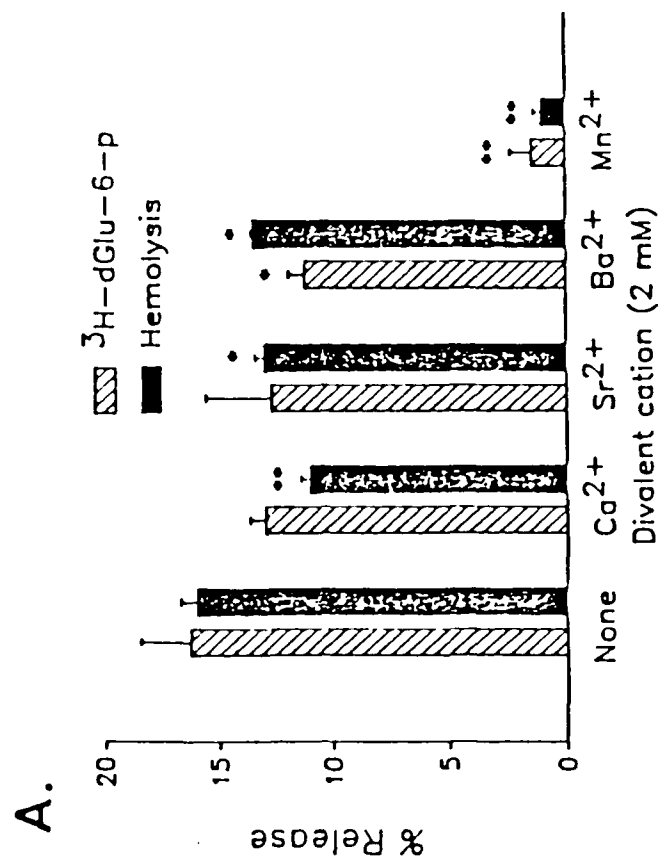


Figure 4

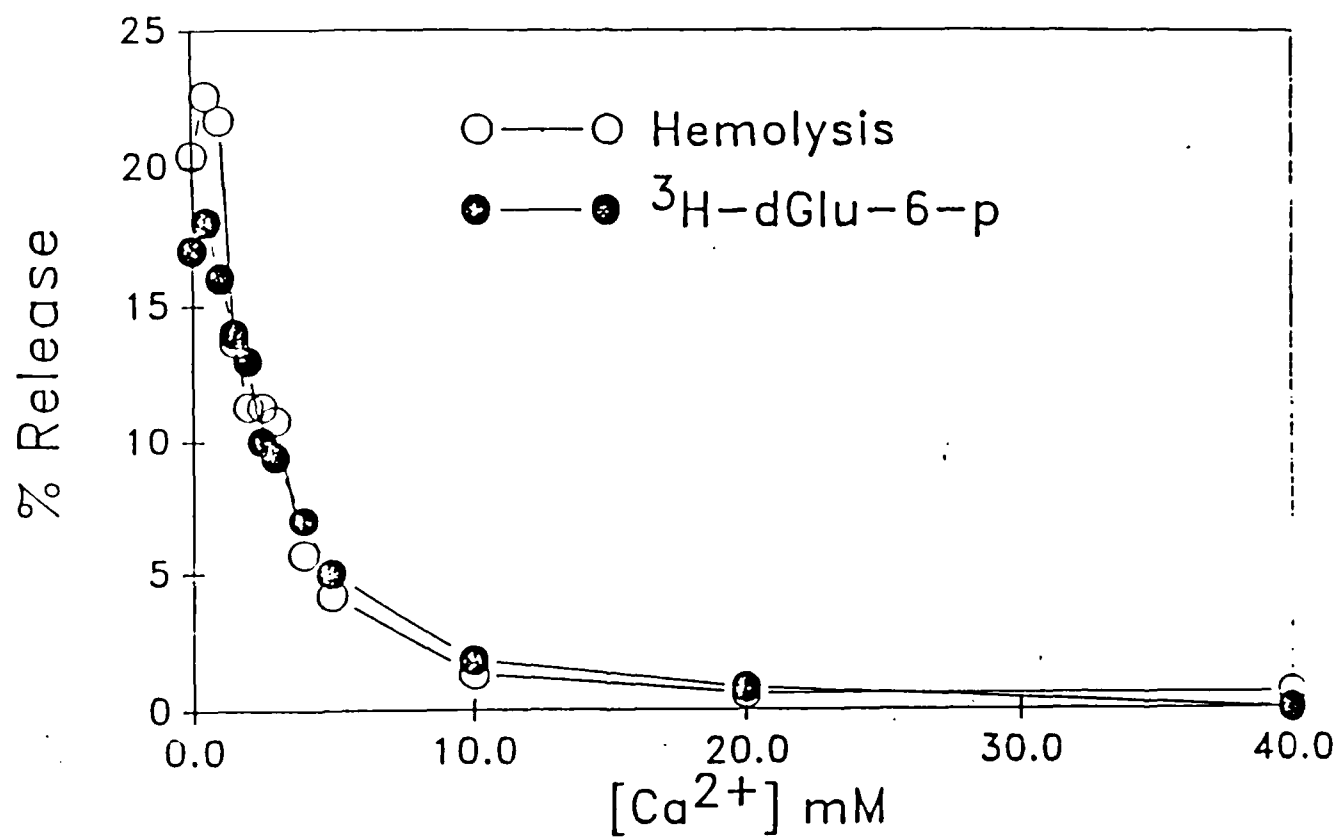


Figure 5

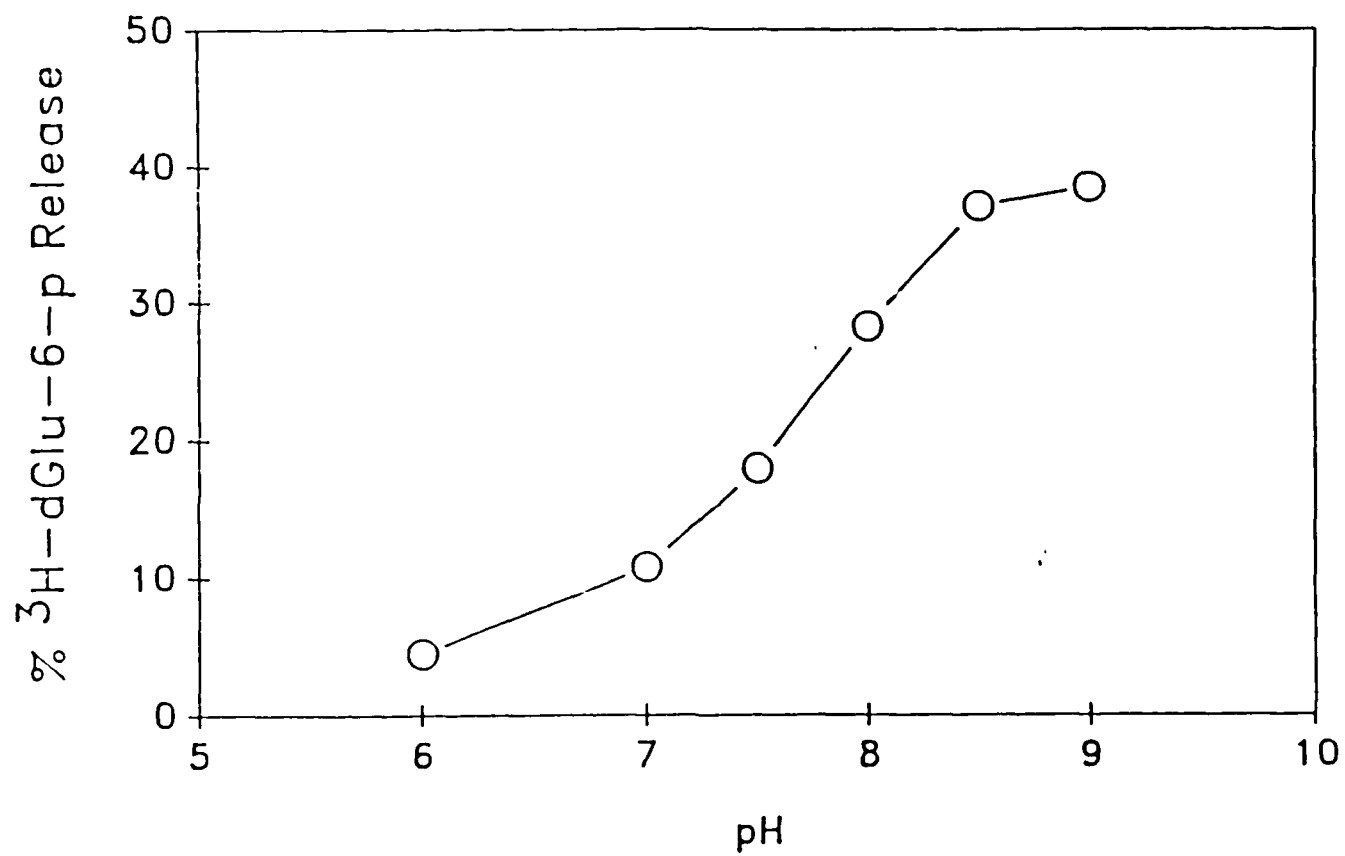


Figure 6

